

EVALUATION OF THE RENAL TOXICITY OF HEME PROTEINS AND
THEIR DERIVATIVES: A ROLE IN THE GENESIS
OF ACUTE TUBULE NECROSIS*

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In many clinical settings acute tubule necrosis is temporally related to breakdown of blood or destruction of tissue (1-6). In the laboratory, myohemoglobinuria, produced in animals by the injection of glycerol, yields a syndrome that is similar in functional features to acute tubule necrosis in man (7-10). This pattern of heme proteinemia followed by acute tubule necrosis has been referred to in the literature as "hemoglobinuric nephropathy" (11, 12). The frequency with which it occurs suggests more than a fortuitous relationship between the release of the heme protein and the induction of the renal lesion.

The mechanism by which hemoglobinemia is associated with injuries in the clinical and experimental setting is uncertain. Suggestions made include the following: (a) heme proteins form casts that plug tubules and damage cells (13-17); (b) heme proteins either cause or are associated with decreased renal blood flow, and the resultant ischemia leads to acute tubule necrosis (18, 19); and (c) heme proteins or some breakdown products directly injure tubules (12, 13, 20, 21).

Any of these three mechanisms, singly or in combination, may play a role in the pathogenesis of renal failure. In this investigation attention has been focused on the effects of heme proteins and/or their breakdown products on the function of renal tubules. Of the parameters studied, depression in organic

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acid and base transport is the earliest and most consistent proximal tubule dysfunction observed. This dysfunction cannot be ascribed to tubule obstruction or ischemia alone. The same tubule dysfunctions are reproduced in vitro with heme proteins. The data indicate that injury to tubule cells results directly from heme proteins. It was found that toxicity is localized to the heme portion of these compounds and that conditions necessary to cause tubule dysfunction in vivo and in vitro are similar. It is postulated that a common mechanism is present in the pathogenesis of tubule dysfunction produced in vivo and in vitro. A parallel is drawn between laboratory and clinical observations.

Materials and Methods

Materials.—The animals used in these experiments were male Sprague-Dawley rats weighing 200–250 g or albino rabbits weighing 2–3 kg.

Hemoglobin was prepared in the manner described by Jaenike (22). Methemoglobin was formed by adding sodium ferricyanide to the hemoglobin. Control injections and control media for studies with methemoglobin contained an amount of sodium ferricyanide equal to that added to the heme protein. Horse heart myoglobin, purified globin, and both human and rabbit albumin were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Ferrihemate¹ was prepared by the method of Morrison and Williams (24). A second preparation was made from crystalline “hematin” (Nutritional Biochemicals) and converted to potassium ferrihemate by alkalization with KOH. Results with either ferrihemate compound were similar, as were the results using rabbit or human albumin. Accordingly, values in this report dealing with either ferrihemate or albumin have been combined. ¹²⁵I-Labeled sodium iodohippurate was obtained as Hippuran from Abbott Laboratories, North Chicago, Ill. and ¹⁴C-tetraethylammonium from Schwarz BioResearch, Inc., Orangeburg, N.Y.

Methods.—Renal cortical slices, 0.3–0.4 mm thick, were prepared with a Stadie-Riggs microtome (25). The weight of the tissue, after blotting on filter paper, was between 50 and 100 mg for rat slices and between 75 and 150 mg for rabbit slices. Incubation of the slices for hippurate and tetraethylammonium transport was performed on a Dubnoff metabolic shaker at 25° ± 0.5°C, shaking at a frequency of 100/min, with 100% oxygen as the gas phase. The slices were incubated for 2 hr in 3.0 ml of Cross and Taggart's medium (26), with a final pH of 7.3–7.5. Isotopically labeled hippurate or tetraethylammonium was placed in the medium at the beginning of incubation to a final concentration of approximately 2×10^{-5} M. For the studies of tissue oxygen consumption, slices were incubated in the above medium at 37°C in a Yellow Springs biological oxygen monitor (27). Incubation of kidney slices for the ammoniogenesis and gluconeogenesis studies was carried out on a Dubnoff metabolic shaker at 37°C, gassed with 95% O₂ and 5% CO₂. The medium was a bicarbonate-buffered Ringer's solution (28). After 2 hr of incubation, the tissues were blotted and weighed. 6 ml of medium was deproteinized with 2 ml of cold 1.2 N perchloric acid. The perchlorate was precipitated in the cold by the addition of a KOH-phosphate buffer mixture to a final pH of 7.4.

Analysis.—Hemoglobin and methemoglobin concentrations were determined by standard methods (29). The hippurate content in slices (S) and medium (M) was determined by isotopic counting, using a well-type γ -scintillation counter, and a slice/medium (S/M) ratio was calculated from the isotopic counts in 1 g of tissue to that of 1 ml of medium. The ¹⁴C-tetra-

¹ The term “ferrihemate”, as suggested by Morrison and Williams (23), is used to denote the products of the splitting of hemoglobin by acid or alkali. “Hematin” is the more general term.

ethylammonium content in slices and medium was determined by means of liquid scintillation. After incubation, slices were suspended in 10% trichloroacetic acid, homogenized, and centrifuged. 1 ml of supernatant fluid was placed in a glass vial containing 20 ml of a scintillation mixture consisting of toluene, Triton X-100, 2,5-diphenyloxazole, and 1,4-bis[2-(5-phenyloxazolyl)] benzene. Portions of medium were treated in a similar manner after deproteinization with 10% trichloroacetic acid. Counting was performed using a Packard Tri-Carb 3320 liquid scintillation counter; samples were corrected back to 100% relative efficiency by means of automatic external standardization from a radium source. Oxygen consumption was measured polarographically, using a Yellow Springs biological oxygen monitor. Results are expressed as microliters per gram (wet weight) per hour. The methods for studying *in vitro* ammoniogenesis and gluconeogenesis were described previously (30). Ammonia was estimated by a modification of the method of Archibald (31), and glucose, by utilizing the glucose oxidase reaction (Glucostat, Worthington Biochemical Company, Freehold, N.J.). Results are expressed as micromoles per gram (wet weight) per hour.

Procedure.—These studies were divided into three separate parts.

I. In vitro renal cortical function after glycerol injections: To induce acute tubule necrosis *in vivo*, rats were given subcutaneous or intravenous injections of 50% glycerol (1 ml/100 g body weight) 16 hr after the removal of drinking water. Controls, which were tested simultaneously, received equal volumes of isotonic saline subcutaneously or intravenously instead of glycerol after a similar period of dehydration. Animals were killed at a given time by a blow to the head, and the kidneys were quickly removed and placed in cold isotonic saline prior to the preparation of kidney slices. In this phase of the study, the abilities of slices from control and glycerol-injected rats to accumulate hippurate and tetraethylammonium, to consume oxygen, to produce ammonia, and to produce glucose were compared.

II. In vitro renal cortical function after methemoglobin injections: In this series of experiments, the toxic effect of methemoglobin injections on renal tubule function was investigated. Rats were deprived of drinking water prior to study, as in part I. Five separate experimental groups, each with its own controls, were set up. In *group 1*, methemoglobin (40 mg/100 g body weight) was injected intraperitoneally 24 hr prior to study. Control animals received an injection of an equal volume of saline. In *group 2*, the left ureter of each rat in the test group was obstructed by a double ligature 24 hr prior to study. Control animals had sham operations consisting of exposure of the left ureter. In *group 3*, the left ureters of the test rats were obstructed in a similar manner. Control animals had the sham operation. Both test and sham-operated animals received methemoglobin (40 mg/100 g body weight) intraperitoneally 24 hr prior to study. In *group 4*, 24 hr before study, the left renal pedicle of the test rats was clamped through a flank incision. After an ischemic period of 1 hr, the clamp was released. Control animals merely had their kidney exposed through the flank incision. In *group 5*, a protocol similar to group 4 was followed, except that both control animals and those with a single ischemic kidney received methemoglobin (40 mg/100 g) following removal of the clip or the exposure of the kidney. In all five groups hippurate accumulation was compared in kidney slices removed from the control and test animals.

III. In vitro renal cortical function after in vitro exposure to heme proteins and derivatives: In the studies of *in vitro* heme protein toxicity, both rat and rabbit kidney slices were used. From week to week, a great variation may take place in the S/M ratios of control kidneys. Since these studies were performed over many months, care was taken to pair a slice from the same kidney of the same animal for control and for test flasks. In this way results are expressed as a percentage of control values in a manner utilized earlier by Koishi (32) to overcome this variability in the procedure. In all studies the control slice was preincubated under the same conditions as the test slice. The medium for test slices contained 1 g heme protein/100 ml. No

heme protein or derivative was added to the medium of the control slices. Hypoxia was produced for both control and test slices when 100% O₂ was replaced by 100% N₂ for 10 min at the beginning of the preincubation. In some experiments, ammonium chloride (1 mM) was added to both control and test media. When an acid environment was desired, HCl was added dropwise to control and test media to bring the pH to 5.4. The slices were preincubated under these conditions for 2 hr at 37°C. At the end of this time both test and control slices were removed from their respective environments and then handled in a similar fashion to determine their ability to accumulate hippurate or tetraethylammonium.

In the studies performed to determine the type of inhibition produced by ferrihemate, the "active" transport of hippurate was studied and the data were plotted against the hippurate concentration in the media in the manner of Lineweaver and Burk (33). Slices incubated in hippurate for 2 hr in a 100% N₂ atmosphere were found to accumulate hippurate such that the slice to medium ratio was 1; i.e., milligrams of hippurate/gram of tissue equaled milligrams of hippurate/milliliter of medium. This uptake was felt to be secondary to diffusion, as there was no possibility of metabolic activity in the slices after this prolonged period of hypoxia. Therefore, "active transport" of hippurate into the tissue was defined as the slice concentration minus the medium concentration ($S - M$).

Statistics of parts I and II were determined by group analysis. Results in part III are expressed in terms of percentage of control values, and statistics were calculated by paired analysis.

RESULTS

Cortical Slice Function after Glycerol Injections.—In agreement with previous findings, animals receiving glycerol intravenously did not show evidence of acute tubule necrosis (9). The blood urea nitrogen in these animals failed to rise above control level 24 hr after the intravenous injection (range, 7–21 mg/100 ml). Subcutaneous glycerol has been shown by many investigators (7–9) to cause renal tubule necrosis. In our studies, the blood urea nitrogen 24 hr after subcutaneous glycerol rose to 67–75 mg/100 ml.

As shown in Table I, renal slices from rats 4 hr after subcutaneous glycerol had a significant impairment of hippurate and tetraethylammonium (TEA)² accumulation when compared with slices of kidneys removed from control animals. The mean uptake of hippurate was 54% of control ($P < 0.001$), and that of TEA was 57% of control ($P < 0.001$). 4 hr after glycerol injections, oxygen consumption was 108% of control, a value not significantly different from control. The ability of slices to produce glucose was significantly increased above control after glycerol injections, while ammoniogenesis was unchanged. The intravenous injection of glycerol did not result in increased gluconeogenesis, nor did it result in any alterations in the kidney's ability to accumulate hippurate or TEA 4 hr later.

In Table II, one can see that 24 hr after intravenous glycerol injections there was no significant alteration in the ability of renal slices to accumulate hippurate and TEA, to consume oxygen, or to produce ammonia and glucose.

² Abbreviation used in paper: TEA, tetraethylammonium.

In the animals receiving subcutaneous glycerol, hippurate and TEA transport showed a greater decrease after 24 hr than after 4 hr, while O₂ consumption was significantly depressed at 24 hr. Although the formation of ammonia was

TABLE I
4 hr after Glycerol or Saline Injections

	Subcutaneous				Intravenous			
	Control (C)	Glycerol (G)	G/C	P	Control	Glycerol	G/C	P
Hippurate S/M	12.5 ± 1.4 (24)	6.7 ± 0.8 (24)	0.54	<0.001	16.3 ± 2.3 (12)	16.0 ± 2.0 (12)	0.98	NS*
TEA S/M	5.1 ± 0.7 (8)	2.9 ± 0.2 (8)	0.57	<0.001				
Oxygen consumption, μ l/g/hr	1.49 ± 0.08 (11)	1.61 ± 0.11 (11)	1.08	NS	1.83 ± 0.17 (8)	1.91 ± 0.36 (8)	1.04	NS
Ammonia formation, μ moles/g/hr	13.8 ± 1.2 (17)	13.1 ± 1.7 (17)	0.95	NS	19.3 ± 1.7 (6)	22.2 ± 1.7 (5)	1.15	NS
Endogenous gluconeogenesis, μ moles/g/hr	7.6 ± 0.9 (11)	19.3 ± 3.1 (11)	2.54	<0.01	10.5 ± 0.7 (6)	13.6 ± 2.4 (5)	1.30	NS

* Not significant.

TABLE II
24 hr Following Glycerol or Saline Injections

	Subcutaneous				Intravenous			
	Control (C) ± SEM	Glycerol (G) ± SEM	G/C	P	Control ± SEM	Glycerol ± SEM	G/C	P
Hippurate S/M	7.6 ± 0.5 (13)	3.0 ± 0.5 (13)	0.39	<0.001	11.9 ± 1.4 (7)	15.1 ± 3.2 (7)	1.27	NS*
TEA S/M	9.8 ± 1.9 (6)	2.7 ± 0.5 (6)	0.28	<0.01				
Oxygen consumption, μ l/g/hr	1.34 ± 0.04 (8)	0.91 ± 0.06 (8)	0.68	<0.001	1.65 ± 0.16 (7)	2.07 ± 0.23 (7)	1.25	NS
Ammonia formation, μ moles/g/hr	32.4 ± 2.4 (14)	24.7 ± 3.1 (14)	0.76	>0.05 < 0.1	28.1 ± 6.4 (7)	22.5 ± 1.9 (8)	0.80	NS
Endogenous gluconeogenesis, μ moles/g/hr	14.0 ± 1.3 (12)	10.4 ± 1.7 (14)	0.74	NS	13.4 ± 1.1 (7)	13.0 ± 1.0 (8)	0.97	NS

* Not significant.

lessened, this decrease did not reach a significant value ($P > 0.05$). Endogenous gluconeogenesis was no longer increased as it was 4 hr after the initiation of acute tubule necrosis.

Hippurate Uptake in Renal Cortical Slices from Rats after Methemoglobin Injections.—Table III portrays the results of studies performed to assess the role of renal obstruction and ischemia in methemoglobin-induced nephropathy as assayed by hippurate transport with the in vitro slice technique.

Methemoglobin alone (group 1): When methemoglobin was injected intraperitoneally into 12 rats (group 1), no significant decrease in hippurate uptake in renal slices, compared with slices from 12 controls, was noted 24 hr later. Sodium ferricyanide alone proved to be nontoxic in vivo or in vitro.

Methemoglobin and obstruction to the ureters (groups 2 and 3): With unilateral obstruction of a ureter for 24 hr in eight rats (group 2), the affected kidney became larger by appearance than the kidneys in eight sham-operated controls. The ability to accumulate hippurate per gram of tissue did not change. Combining methemoglobin injections with 24 hr of ureteral blockage in eight other

TABLE III
Effects of Methemoglobin on Hippurate Uptake

Group	Condition	Control (C) \pm SEM	Test (T) \pm SEM	T/C	P
1	Methemoglobin alone	8.2 \pm 1.1 (12)	9.0 \pm 0.9 (12)	1.10	NS*
2	Ureter obstructed	6.5 \pm 0.3 (8)	6.7 \pm 0.3 (8)	1.03	NS
3	Ureter obstructed, methemoglobin given	6.6 \pm 0.3 (8)	6.9 \pm 0.3 (8)	1.05	NS
4	Renal ischemia, 1 hr	6.3 \pm 0.8 (12)	11.7 \pm 1.2 (12)	1.86	<0.01
5	Renal ischemia, 1 hr; methemoglobin given	10.7 \pm 0.8 (25)	7.4 \pm 0.6 (24)	0.69	<0.01

* Not significant.

animals (group 3) also failed to affect proximal tubule function within 24 hr of the injection, as measured by hippurate accumulation.

Methemoglobin and renal ischemia (groups 4 and 5): Clamping the right kidney pedicle for 1 hr resulted in a cyanotic appearance to the kidney, which remitted when the clamp was removed (group 4). The change in appearance of the kidney, from the cyanotic blue color to pink, indicated the reestablishment of flow. The result of 1 hr of ischemia in 12 rats was an increase in hippurate accumulation significantly greater in the ischemic kidneys when compared to the kidneys from the 12 sham-operated animals. When methemoglobin injections were combined with unilateral renal ischemia of 1 hr duration in 25 rats (group 5), hippurate accumulation decreased when compared to kidneys from 24 controls.

The In vitro Effect of Heme Proteins and Their Derivatives on Hippurate Uptake of Rabbit Kidney Slices.—At the physiological pH of 7.4, all proteins tested, with the exception of myoglobin, produced no significant changes in hippurate uptake (Fig. 1). The preincubation of kidney slices in albumin, a protein of

molecular weight similar to oxyhemoglobin and methemoglobin, did not result in a decrease in hippurate uptake when compared to control kidney slices incubated without albumin [$109\% \pm 14.4$ (SEM)]. Oxyhemoglobin and methemoglobin showed similar findings when compared with controls [$112\% \pm 15.0$ (SEM) and $104\% \pm 6.3$ (SEM)]. Slices preincubated in myoglobin, a protein roughly one-fourth the molecular weight of the others, decreased hippurate ac-

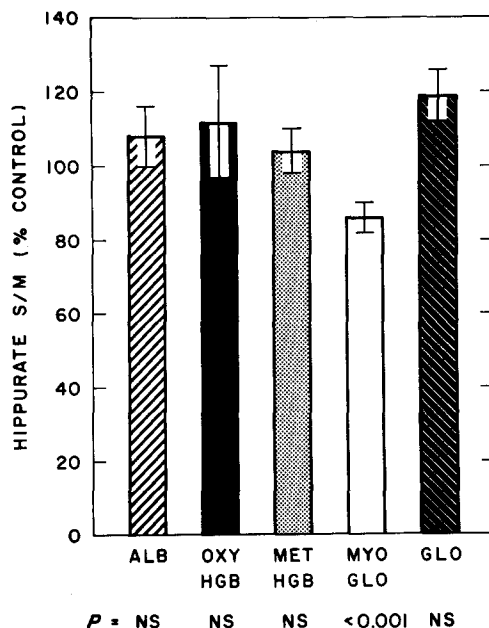


FIG. 1. Hippurate transport after preincubation in 1 g/100 ml protein solutions. ALB albumin; OXY HGB, oxyhemoglobin; MET HGB, methemoglobin; MYO GLO, myoglobin; GLO, globin; NS, not significant. Results are expressed as slice to medium (S/M) ratios (percentage of control) as described in the text.

cumulation to $86\% \pm 4.0$ (SEM) of control ($P < 0.001$). Slices preincubated in globin had hippurate uptakes $119\% \pm 7.1$ (SEM) of control.

When conditions associated with the production of hemoglobinuric nephropathy in vivo were simulated in vitro (4), alterations in hippurate accumulation by rabbit renal cortical slices were produced. With a 10 min interval of hypoxia to both control and test slices during the preincubation time (Fig. 2), a significant decrease in hippurate uptake of those slices preincubated in the heme proteins was seen. Under these conditions, there was a decrease in hippurate uptake to $68\% \pm 6.9$ (SEM) of the simultaneously run controls ($P < 0.01$) when slices were incubated with oxyhemoglobin, to $59\% \pm 3.2$ (SEM) of controls ($P < 0.001$ with methemoglobin, and to $72\% \pm 4.9$ (SEM) ($P < 0.01$)

with myoglobin. Albumin and globin had no significant effect on hippurate uptake, even under hypoxic conditions. The effect of increasing the concentrations of ammonia in the medium in combination with heme proteins was studied. When 1 mM NH_4Cl was added to preincubation media of control slices and those containing the heme proteins, methemoglobin and myoglobin depressed hippurate accumulation to $74\% \pm 4.1$ (SEM) ($P < 0.001$) and $77\% \pm$

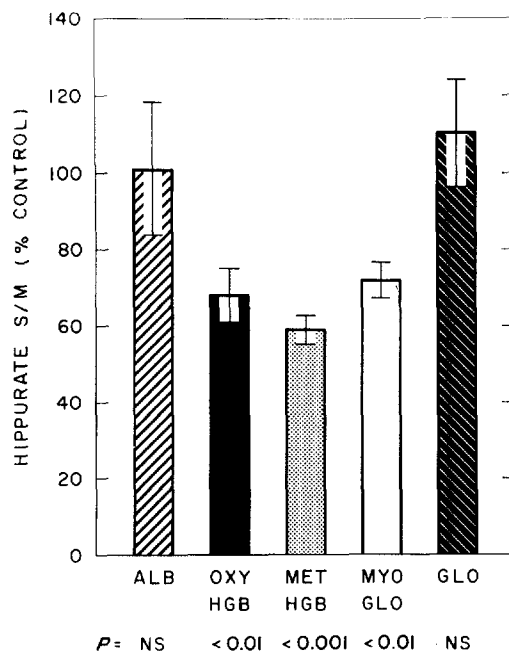


FIG. 2. Hippurate transport after 10 min of hypoxia and incubation in 1 g/100 ml protein solutions. Abbreviations are the same as in Fig. 1.

6.9 (SEM) ($P < 0.05$), respectively (Fig. 3). Although the hippurate accumulation after preincubation in oxyhemoglobin was decreased to $92\% \pm 7.4$ (SEM) of control, this was not significant. Globin alone had no effect. It was interesting to note that in the presence of albumin, hippurate accumulation was greater in the presence of ammonia than controls [$140\% \pm 16.9$ (SEM)], although this figure did not reach a significant value ($0.1 < P > 0.05$).

The last model tested was incubation in an acid environment of pH 5.4 (Fig. 4). Again, when compared with control slices incubated at the same pH, albumin and globin produced no significant changes in hippurate uptake, while oxyhemoglobin [$85\% \pm 4.2$ (SEM)] ($P < 0.025$) and myoglobin [$63\% \pm 7.7$ (SEM)] ($P < 0.02$) decreased uptake when compared to controls.

Like albumin, globin did not depress hippurate accumulation under any

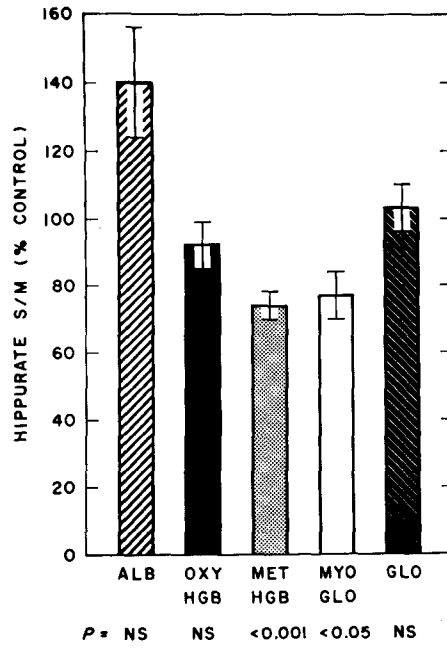


FIG. 3. Hippurate transport after incubation in NH_4Cl (1 mM) and in 1 g/100 ml protein solutions. Abbreviations are the same as in Fig. 1.

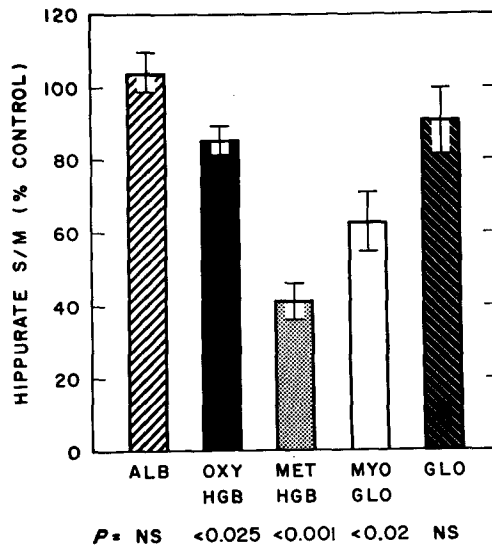


FIG. 4. Hippurate transport at pH 5.4 and in 1 g/100 ml protein solutions. Abbreviations are the same as in Fig. 1.

condition investigated. Therefore, the next series of experiments was designed to see if the heme portion (ferrihemate) of these proteins was responsible for these findings. As can be seen in Fig. 5 A, ferrihemate alone, without other insults, caused a depression in hippurate uptake when compared to control slices. The amount of depression varied directly with the concentration of ferrihemate. Fig. 5 B illustrates that there was increasing depression of hippurate uptake with increasing preincubation times of the slices in the medium

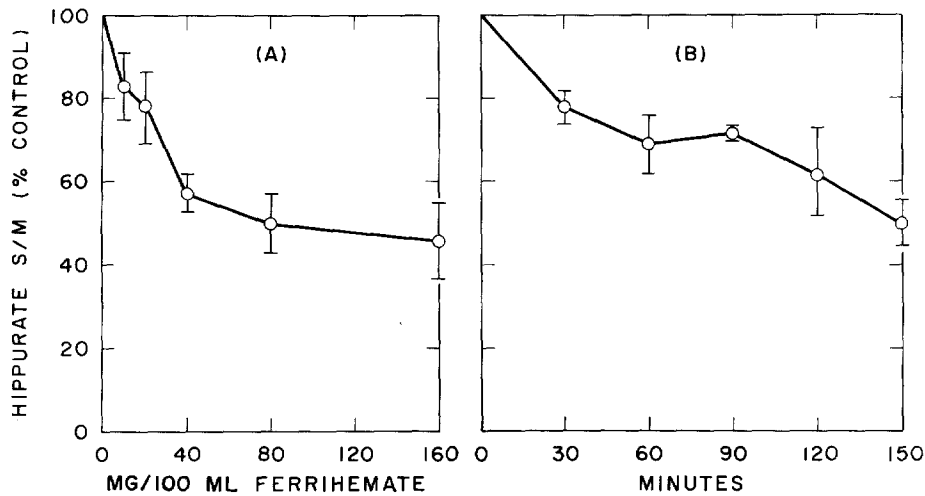


FIG. 5. A. Slices in control medium were paired against slices preincubated in ferrihemate at different concentrations. After 2 hr of preincubation the hippurate S/M ratios were determined. Results are expressed as test slice S/M ratios divided by control slice S/M ratios \times 100. B. Paired slices were preincubated in control medium or medium containing 40 mg/100 ml ferrihemate for a given length of time. The hippurate S/M ratios were then determined as percentage accumulation in test slices related to control slices.

containing 40 mg/100 ml ferrihemate, the approximate amount present in a 1 g/100 ml solution of hemoglobin. Consistent with the *in vivo* studies, no significant effect of ferrihemate *in vitro* on oxygen consumption or gluconeogenesis was noted in over 30 slice experiments.

When albumin was added to the ferrihemate medium, the depression in hippurate transport produced by ferrihemate was not seen (Table IV). Thus, ferrihemate alone depressed hippurate uptake, but neither albumin alone nor the ferrihemate-albumin medium had a depressant effect. However, when exposed to 10 min of hypoxia, the tissue slices incubated in the ferrihemate-albumin combination showed a significant depression of hippurate transport compared to control slices incubated in albumin alone.

To test whether hippurate accumulation could be used as a test of dysfunc-

tion of the tubules in the presence of ferrihemate or merely reflected competitive inhibition between ferrihemate and hippurate, the following studies were performed. Slices were incubated in increasing concentrations of hippurate to

TABLE IV
Effects of Ferrihemate on Hippurate Uptake

Group	Condition	No. of Experiments	Control S/M	Test S/M	Control S/M	P
1	Ferrihemate	20	15.0 ± 1.8	8.5 ± 2.1	%	<0.001
2	Ferrihemate + albumin	8	10.1 ± 1.0	9.8 ± 1.1	62 ± 4.2	NS*
3	Ferrihemate + albumin + hypoxia	7	17.4 ± 1.6	12.8 ± 2.4	97 ± 8.0	<0.05

* Not significant.

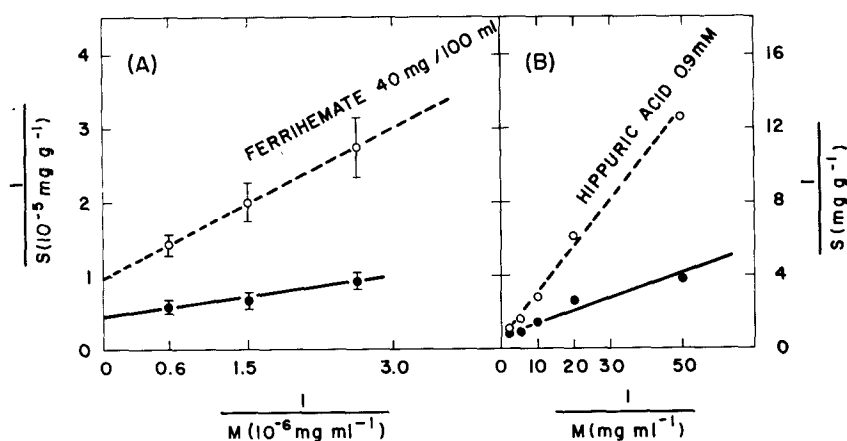


FIG. 6. A. Lineweaver-Burk plot of ferrihemate (40 mg/100 ml) inhibition as a function of hippurate concentration. Means and standard errors of the mean for three experiments are shown. B. Lineweaver-Burk plot of hippuric acid (0.9 mM) inhibition as a function of *p*-aminohippurate concentration.

determine whether the inhibition from ferrihemate could be overcome. In Fig. 6 A the plot for "active" hippurate transport in the presence of ferrihemate was typical of noncompetitive inhibition (33). A similar plot made under these same conditions for a known competitive inhibitor is presented for comparison in Fig. 6 B.

In Fig. 7, one can see the effects on hippurate accumulation of incubation in *p*-aminohippurate, a known competitive inhibitor of hippurate, and the sub-

stance under study, ferrihemate. In the presence of *p*-aminohippurate, the hippurate S/M ratio remained depressed at the same level over 4 hr. In the presence of ferrihemate, the S/M ratios progressively decreased with time.

To test whether ferrihemate would affect transport systems other than the organic acid system, TEA transport was studied. After preincubation in 40 mg/100 ml of ferrihemate for 2 hr, TEA accumulation was significantly de-

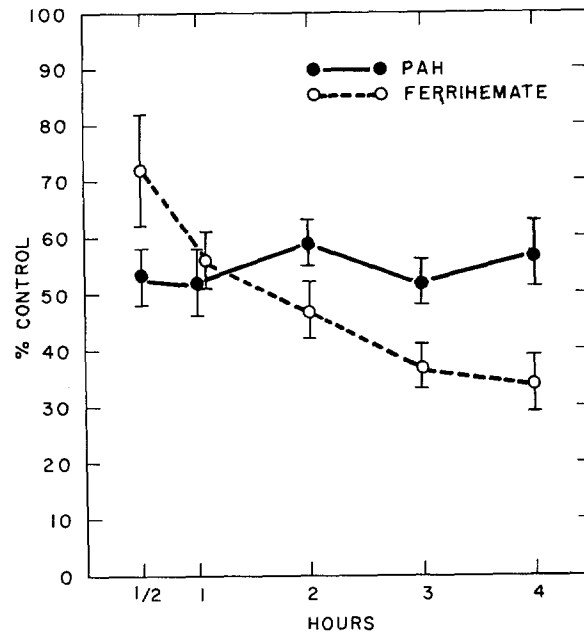


FIG. 7. S/M ratios expressed as percentage of paired control slices for hippurate accumulation in the presence of ferrihemate (40 mg/100 ml) and *p*-aminohippurate (PAH, 2×10^{-5} M). Ratios were obtained after a given period of incubation, as indicated on the abscissa.

pressed when compared with control slices handled in a similar manner [$23\% \pm 4.0$ (SEM)] ($p < 0.001$). The actual mean S/M ratio for the controls was 5.5 ± 0.8 (SEM), and for the test slices it was 1.1 ± 0.2 (SEM). This corresponded with the depression in organic base transport noted early in the course of glycerol-induced acute tubule necrosis.

DISCUSSION

Acute tubule necrosis following subcutaneous injections of glycerol is a well studied model of hemoglobinuric nephropathy (7-10). While the pathological changes are documented in this model (7, 9), the physiological changes are not well known. As a first need, it was necessary to establish the correlates of physiological and pathological injury.

It is known that rats have myohemoglobinuria hours after subcutaneous glycerol injection (7-10). The proximal convoluted tubules show swelling, pallor, and granules of hemoglobin within cells at 4 hr. From the data presented in Table I, it can be seen that at 4 hr, among the various physiological functions studied, only hippurate and tetraethylammonium transport was significantly decreased from control values. On the other hand, at 24 hr, following subcutaneous injections, hippurate and tetraethylammonium transport and oxygen consumption in kidney cortical slices were decreased significantly below control values. This time-dependent disparity between depression of hippurate accumulation and depression of oxygen consumption after insult has been noted in a previous study (30).

That glycerol itself is not responsible for the changes noted in this model can be seen from the data in animals given glycerol intravenously. At 24 hr the blood urea nitrogen had not changed, and hippurate transport, gluconeogenesis, ammoniogenesis, and oxygen consumption of test slices were not significantly different from control slices.

To evaluate further the correlation between hippurate transport and acute renal injury, ischemia coupled with heme protein injection was used as a second model of acute tubule necrosis. Carroll et al. (9) studied the pathological changes noted in this model. They showed that injections of hemolyzed blood would cause acute tubule injury when coupled with ischemia, and that hemolyzed blood alone or combined with ureteral obstruction caused no morphological damage to the kidney. The data presented in Table III summarize the physiological studies on models patterned after Carroll's groups. It can be seen that depression in hippurate transport occurs only under the conditions which produce morphological changes indicative of cellular injury in the kidney. When rats were subjected to ureteral obstruction alone, ureteral obstruction plus methemoglobin injections, or 1 hr of renal ischemia alone, depression in hippurate transport was not observed.

The reason for the significant increase in hippurate transport following 1 hr of ischemia is not entirely clear. It may relate to the fact that slices removed from ischemic kidney tissue under stimulus to hypertrophy may accumulate more hippurate. Such a phenomenon has been described as occurring in slices removed from hypertrophying kidney as early as 24 hr after uninephrectomy (34).

From these initial studies several conclusions are drawn. First, early in acute tubule injury there is depression in hippurate transport. Second, neither ischemia nor obstruction alone produces a similar depression in hippurate transport. Therefore, in both models studied there must be another factor which induces cellular injury. Heme proteinemia is common to both the experimental models of acute tubule necrosis. In many diverse clinical cases of acute tubule necrosis, heme proteinemia is also noted (12, 13, 15, 21). Since it was established that hippurate transport could be used as an index of early tubule injury, the effects of various heme proteins on this transport system were investigated. The heme proteins studied were hemoglobin, methemoglobin, and myoglobin. In addition, studies were performed with albumin and globin.

Hemoglobin or methemoglobin preincubated with renal slices did not affect hippurate transport. However, when combined with a brief episode of hypoxia, the presence of these heme proteins in the medium caused a significant decrease in hippurate accumulation. A correlation between these studies in Fig. 2 and those in Table III can be seen.

Incubation of slices in either globin or albumin alone produced no significant change from control in hippurate transport. That globin in any of the *in vitro* settings caused no change in hippurate accumulation suggested that the toxicity of heme proteins resided in the heme portion of these compounds. This suggestion was verified when preincubation of slices with ferrihemate in the medium showed consistent depression of hippurate transport.

The depression of hippurate transport by ferrihemate may be interpreted in two ways: either the ferrihemate acts as a noxious agent to the cell and the first effects are measured in the hippurate transport system, or the ferrihemate acts as a competitive inhibitor of organic acid transport. Several studies favor the acceptance of the former of these two possibilities.

When hippurate uptake is studied in the presence of ferrihemate and a known competitive inhibitor, the Lineweaver-Burk plots of the kinetics are not similar for the two substances. The ferrihemate curve is not like that of a competitive inhibitor. Furthermore, when the depression of hippurate transport is studied in the presence of ferrihemate, the percentage depression as a function of time is seen to increase, unlike the percentage depression with a known competitive inhibitor. In the latter case the percentage depression as a function of time is seen to be constant.

Finally, ferrihemate alters another transport system, that of organic bases, as represented by tetraethylammonium accumulation. Like organic acid transport, this system is depressed early in the course of acute tubule necrosis following glycerol injection. It is hardly likely that the same substance would be a competitive inhibitor of both organic acid transport and organic base transport.

Why is ferrihemate toxic under any condition whereas heme proteins (hemoglobin-methemoglobin) prove depressive only in certain settings? Is there a relation between these experimental situations and clinical cases in which certain preexisting conditions are necessary to produce acute tubule necrosis in the presence of heme proteinemia?

The hypothesis that is most consistent with the data presented in this study involves the concept of cell membrane selectivity. As long as cellular metabolism functions adequately, there is cell membrane integrity. With depression of cellular metabolism, there is a breakdown in membrane integrity and selectivity, with the result that heme proteins enter the cell. Normally the large molecular size of heme proteins prevents their entrance into areas of the tubule cells where they may be damaging.

To evaluate this hypothesis further, several other models were used to study the effects of heme proteinemia in slices placed under stress. *In vitro*, ammonia has been shown to depress oxidative metabolism. According to the data presented in Fig. 1, neither hemoglobin alone nor methemoglobin alone affects slice hippurate accumulation compared to control. NH_4Cl at 1 mM also has little effect on hippurate accumulation in slices (30). However, when both NH_4Cl and methemoglobin are placed in the medium together, there is a marked depression of slice hippurate accumulation (Fig. 3). Fig. 4 shows the results of incubation of slices at low pH. Again it is noted that under these conditions hemoglobin and methemoglobin depress hippurate uptake. That ferrihemate is injurious without cellular membrane damage is due to the difference in size of the ferrihemate and other hemoproteins. Ferrihemate, with a molecular weight of approximately 670, is much smaller in size than hemoglobin, with a molecular weight of approximately 68,000.

Strengthening the hypothesis that intact membranes and the molecular size of heme proteins may prevent their entrance into areas of the cell where they may exert toxicity is the finding that the addition of albumin to the medium containing ferriheme negates the depression in hippurate transport seen with ferriheme alone. In the presence of albumin, ferriheme combines to form a larger molecular, methemalbumin (13). However, with 10 min of hypoxia, methemalbumin also is associated with hip-

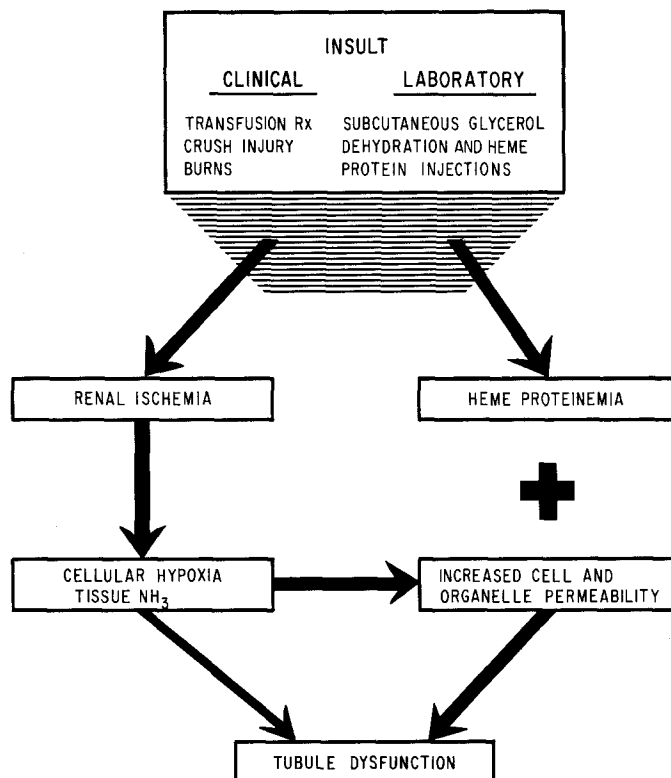


FIG. 8. Theoretical model for the development of acute tubule necrosis

purate depression to the same extent as with other heme proteins of similar molecular weight.

Another explanation may be put forth for increased toxicity of heme proteins in the medium of pH 5.4 other than tissue injury with decreased membrane selectivity. Dissociation of the heme protein into heme and globin moieties takes place at this pH (35). This dissociation would permit the subsequent entrance of ferriheme into the cell.

Finally, the ability of myoglobin to suppress hippurate transport in control medium can be explained by at least two possibilities. First, its smaller molecular size might

allow it to cross even unaltered cell membranes; second, because of its smaller molecular weight, a 1 g/100 ml solution would contain approximately 4 times more ferrihemate than hemoglobin or methemoglobin solutions of 1 g/100 ml.

From the *in vitro* and *in vivo* studies presented, the following model is proposed for the pathogenesis of "hemoglobinuric nephropathy" (Fig. 8). By one of several techniques, dehydrated rats are subjected to a transient period of renal ischemia (9). This ischemia results in both renal hypoxia and elevated renal tissue concentrations of ammonia. Either one or both lead to alterations in tissue energetics. These alterations affect membrane selectivity of the renal cells in such a way as to allow entrance of heme proteins into vital areas of the cells. The ferrihemate, in conjunction with ischemia, produces acute tubule dysfunction, cellular injury, and necrosis.

The model proposed not only would explain the experimental data presented, but could be used to explain the renal failure seen following crush syndrome, burns, and mismatched blood transfusion reactions. This model may be the counterpart of the clinicopathological situation in which the combined effects of hypoxia and free hemoglobin result in the production of acute tubule necrosis (4). It is not mutually exclusive of the theory that tubule obstruction plays some role in the pathogenesis of acute tubule necrosis.

SUMMARY

This investigation studies the toxicity of heme proteins and/or their breakdown products on renal function. Heme proteinemia precedes acute tubule necrosis at a frequency great enough to suggest a causal relationship between the two events. Physiological and metabolic functions of kidney slices are investigated in several models of acute tubule necrosis. Organic acid and organic base transport is depressed earliest. These alterations in tubule function cannot be explained by ischemia or obstruction alone. Heme proteinemia in rats or incubation of renal slices in medium containing heme proteins yields several interesting observations. Neither *in vivo* or *in vitro* do hemoglobin and methemoglobin alone produce a depressive effect on the transport systems studied. However, parallel to many clinical situations, when such secondary insults as hypoxia and elevated ammonia concentrations are included in the experimental design, transport functions are depressed. Ferrihemate, a molecule smaller than hemoglobin or methemoglobin, depresses transport function without secondary insults. From these studies it is concluded that heme proteins play a role in tubule dysfunction seen in acute tubule necrosis. A model is presented that collates these data with other factors known to play a part in the pathogenesis of this renal syndrome.

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